

Identification of potential circular RNA biomarkers in lung adenocarcinoma: A bioinformatics analysis and retrospective clinical study

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Abstract. Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-associated mortality. Lung adenocarcinoma (LAC) is the most prevalent pathological subtype of NSCLC and accounts for ~40% of all lung cancer mortalities. There remains an urgent demand for the identification of novel biomarkers for the diagnosis and development of therapeutic strategies for LAC. In the present study, the profiles of the differentially-expressed circular RNAs (circRNAs) in LAC tissues compared with those in their corresponding non-cancerous tissues were obtained after analyzing the circRNA microarray dataset GSE101586. The expression pattern of the indicated circRNAs in the LAC tissues were subsequently verified using reverse transcription-quantitative PCR (RT-qPCR). The potential prognostic significance of these circRNAs in patients with LAC were then analyzed in a retrospective clinical study. A circRNA-microRNA (miR or miRNA)-mRNA regulatory network in LAC was established by using Cytoscape. In addition, a protein-protein interaction (PPI) network was plotted using the Search Tool for the Retrieval of Interacting Genes/Proteins and visualized through Cytoscape. The prognostic value of the hub genes found was then analyzed based on the Gene Expression Profiling Interactive Analysis database. In total, four differentially-expressed circRNAs were obtained from the GSE101586 microarray dataset, three of which (hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666) were confirmed by RT-qPCR to be highly

expressed in LAC tissues. This retrospective clinical study revealed that higher expression levels of these three circRNAs were associated with poorer prognoses in patients with LAC. In addition, siRNA-mediated knockdown of these circRNAs was found to inhibit cell proliferation, migration and invasion in LAC cells. Following analysis of the molecular mechanism underlying these circRNAs, eight miRNAs, namely miR-520f, miR-1261, miR-1270, miR-620, miR-188-3p, miR-516b, miR-940 and miR-661, were identified with potential binding sites for these three circRNAs. Subsequently, 232 overlapped genes from the 795 upregulated genes in the LAC samples from The Cancer Genome Atlas database and 7,829 predicted target genes of the list of eight aforementioned miRNAs were obtained. A circRNA-miRNA-mRNA network was then constructed. A PPI network was established, with six hub genes, namely kinesin family member (KIF) 2C, KIF18B, maternal embryonic leucine zipper kinase, baculoviral IAP repeat-containing 5, polo-like kinase 1 and cytoskeleton-associated protein 2-like, determined from this network. Higher expression levels of each of these hub genes were found to be associated with poorer prognoses of patients with LAC. To conclude, data from the present study suggested that circRNAs hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 have the potential to be viable biomarkers and therapeutic targets for LAC.

Introduction

Despite the recent progress in the development of targeted therapies, lung cancer remains to be the most prevalent malignancy and the leading cause of cancer-associated mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 80-85% of all types of lung cancer, where lung adenocarcinoma (LAC) is the most prevalent pathological subtype and accounts for ~40% of all lung cancer-associated mortality (2). Although comprehensive therapeutic strategies, including surgery and targeted therapies, have improved clinical outcome over the past number of decades, the 5-year survival rate of LAC remains at only 18% (3). The reasons for this poor prognostic rate from advanced LAC include low diagnosis rates and the absence of effective therapeutic targets.

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Therefore, it would be of great value to identify novel specific and sensitive biomarkers and therapeutic targets for patients with LAC.

Circular RNAs (circRNAs) are a novel class of endogenously-spliced RNAs that have been previously found to regulate cancer progression (4,5). Due to their covalently closed loop without a 5'-3' poly-adenylation end, circRNAs possess the ability to resist the activities of exonucleases and remain highly stable, suggesting that they have potential to serve as cancer biomarkers and therapeutic targets (6-10). Accumulating evidence has demonstrated that circRNAs are involved in the development of LAC (11-13). One key molecular function of circRNAs is that they can function as competing endogenous RNAs (ceRNAs) to sponge microRNAs (miRNAs), leading to the enhancement of the expression of genes normally suppressed by miRNAs. The circRNA circ-cysteine-rich transmembrane bone morphogenetic protein regulator 1 inhibits the invasion and metastasis of LAC cells by serving as the ceRNA of miR-182/miR-93 (14). In addition, another circRNA, *has_circ_0001946*, promotes LAC cell proliferation by sponging miR-135a-5p (15). In another study, circ-zinc finger protein 609 promotes the proliferation of LAC by targeting miR-1224-3p/ETS translocation variant 1 signaling (16). Taken together, these previous findings suggest that circRNAs serve crucial roles in regulating the physiology of LAC and have the potential to serve as biomarkers and therapeutic targets of LAC.

In the present study, by analyzing the GEO database, three circRNAs were identified to be highly expressed in LAC tissues compared with the paired-matched adjacent non-cancerous tissues. A retrospective clinical study revealed that higher expression levels of these three circRNAs indicated poorer prognoses in patients with LAC. To unravel the potential biological mechanism of these circRNAs, their target miRNAs and possible mRNAs affected downstream were screened, following which a circRNA-miRNA-mRNA regulatory network was constructed. A protein-protein interaction (PPI) network was subsequently established and six hub genes were eventually identified. A retrospective study using the Gene Expression Profiling Interactive Analysis (GEPIA) database showed that higher expression levels of each hub gene were associated with poorer overall survival in patients with LAC. Taken together, through bioinformatic analysis and retrospective clinical study, these findings revealed a number of novel circRNAs that could be important for LAC pathophysiology. In addition, they provided insights into the molecular mechanism that regulate the progression of LAC with respect to the circRNA-miRNA-mRNA network.

Materials and methods

Datasets. The circRNA expression profile of GSE101586 was obtained from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), which included five pairs of matched LAC tissues (GSM2706428, GSM2706429, GSM2706430, GSM2706431 and GSM2706432) and adjacent non-cancerous tissues (GSM2706423, GSM2706424, GSM2706425, GSM2706426 and GSM2706427). The tissues were obtained from five female patients with LAC with no smoking history, and the

physiology of the tissues were confirmed by pathologists. The mRNA data of the LAC tissues was downloaded from The Cancer Genome Atlas (<https://portal.gdc.cancer.gov>).

Clinical samples. In total, 60 cases of LAC tissues in the tissue bank were obtained from the patients who underwent surgery in the Fourth hospital of Hebei Medical University (Shijiazhuang, China) between January 2016 and June 2016. A total of 20 pairs of LAC and adjacent normal tissue (5-cm from the tumor tissue) were collected from the above 60 cases. The clinicopathological characteristics and survival status of the patients with LAC were obtained from the follow-up data. (The duration of follow-up was between January 2016 and June 2021). The inclusion criteria were: i) All patients were diagnosed LAC through pathology and ii) All patients did not receive any therapy before surgery. The clinicopathological data for the patients are presented in Table SI. The human tissues were obtained with written informed consent, and the present study was approved by The Clinical Research Ethics Committee of The Fourth hospital of Hebei Medical University (approval no. 2021KY157).

Differentially-expressed circRNAs and mRNAs. The limma package in R (v3.4.1; <https://bioconductor.org/packages/release/bioc/html/limma.html>) was used to identify the differentially expressed circRNAs between the LAC tissues and the adjacent non-cancerous tissues from the GEO101586 dataset using the criteria of $P < 0.05$ and $|\log_2 \text{fold change (FC)}| > 1$. The 'edgeR' package (v3.52; <http://bioconductor.org/packages/edgeR/>) was used to screen for the differentially-expressed mRNAs using thresholds of $|\log_2 \text{FC}| > 3$ and $P < 0.01$. The miRNAs-targeted mRNAs were predicted based on the miRWalk software (v2.0; <http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/>).

Cell culture. LAC cell lines H1299 and H1975 were obtained from Chinese Academy of Sciences Cell Bank were cultured in RPMI-1640 media (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Cells were cultured at 37°C under 5% CO₂.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from 80% confluent cells and tissues were isolated by TRIzol® Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The cDNA was prepared from total RNA according to the GoScript Reverse Transcription System (Promega Corporation) protocol. qPCR analysis was performed using the Promega GoTaq qPCR Master Mix (Promega Corporation). GAPDH was used as the mRNA internal reference. The primer sequences for all qPCR reactions are shown in Table SII. The PCR cycling conditions was as follows: Denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec and extension at 72°C for 30 sec. Three repeat wells were set. The relative expression levels were calculated using the 2^{- $\Delta\Delta\text{Ct}$} method (17). The sequencing of PCR products were obtained from Sangon Biotech Co., Ltd. RNase R treatment was carried out for 15 min at 37°C using RNase R 3 U/mg.

siRNA transfection. siRNA for negative control and siRNAs targeting the junction sites of circ-0006220, circ-0072088, and

circ-0001666 were designed and synthesized by Guangzhou RiboBio Co., Ltd. The sequences of these siRNAs are listed in Table SIII. H1299 cells were transfected with above siRNAs when reached 70-80% confluence by using HiPerFect Transfection Reagent (Qiagen GmbH) based on the manufacturer's instruction. The final siRNA concentrations were 50 nmol/l. Following transfection, cells were cultured at 37°C for 72 h.

Cell Counting Kit-8 (CCK-8) (MCE®) assay. H1299 cells were first seeded at a cell density of 5×10^3 cells/well into 96-well plates. After attachment overnight, CCK-8 assays were performed after 24, 48 and 72 h. In brief, 10 μ l CCK-8 solution (cat. no. HY-K030; MedChemExpress) was added into each well. After 1 h incubation at 37°C, the absorbance readings for each well were performed at 450 nm using the microplate reader (Tecan Group, Ltd.).

Transwell migration and Matrigel invasion assays. The migration and Matrigel invasion assays were performed in 6.5-mm Transwell chambers for migration assays or Matrigel pre-coated (at 37°C for 24 h) chambers for invasion assays based on the manufacturer's protocol (BD Biosciences). The cell suspensions of different groups were added to the upper chambers at a density of 5×10^4 cells/well and incubated for 24 h. The migratory and invasive cell numbers were then quantified in five random fields per chamber under the inverted microscope at x100 magnification.

Prediction of circRNA-miRNA binding sites. Prediction of interactions between circRNAs and miRNAs was performed using the CircInteractome database (<https://circinteractome.irp.nia.nih.gov/>). miRNAs with a context score percentile ≥ 98 were eventually selected.

Construction of the circRNA-miRNA-mRNA regulatory network. The ceRNA regulatory network was established according the possible interactions among the four differentially-expressed circRNAs, eight miRNAs predicted to be targeted by these circRNAs and the 232 overlapped mRNAs from the list of predicted target genes and the upregulated genes in LAC. Cytoscape 3.7.1 software (v.3.7.1; <https://cytoscape.org/>) was used to visualize the established ceRNA regulatory network.

Establishment of the PPI regulatory network and identification of hub genes. A PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) software (version 11.5; <http://string-db.org/>) and visualized using the Cytoscape 3.7.1 software (<https://cytoscape.org/>). Additionally, suspected hub genes with high predicted degrees of interactions from the PPI network were screened using the CytoHubba plugin in Cytoscape.

Verification of hub gene expression and survival using the GEPIA database. The mRNA expression levels of the hub genes and their association with survival were assessed using the web-based GEPIA database (<http://gepia.cancer-pku.cn/>) with the settings of $P \leq 0.05$ and $|\log_2 FC| \geq 1$.

Immunohistochemistry. The 5- μ m, paraffin-embedded slides were deparaffinized in xylene, rehydrated using a decreasing

alcohol gradient and washed with 1X PBS (pH 7.2) three times at 5 min each. The sections were then heated in a microwave oven for 5 min in 10 mmol/l Na-citrate buffer (pH 6.0) for antigen retrieval and washed with 1X PBS. The sections were immersed in 0.3% hydrogen peroxide in methanol for 20 min to suppress endogenous peroxidase activity. After further washing with 1X PBS, the sections were incubated in 10% normal goat serum (cat. no. SP-9001 or SP-9002; OriGene Technologies, Inc.) at room temperature in a humidified chamber for 30 min to prevent nonspecific immunoglobulin binding. The sections were then treated with the 1:100-diluted KIF2C antibody (cat. no. TA503320; OriGene Technologies, Inc.), KIF18B (cat. no. ab121798; Abcam), CKAP2L (cat. no. ab122617; Abcam), PLK1 antibody (cat. no. TA500383; OriGene Technologies, Inc.), MELK antibody (cat. no. ab129373; Abcam), BIRC5 antibody (cat. no. TA301427; OriGene Technologies, Inc.) at 4°C overnight. Normal IgG in place of the primary antibody served as the negative control. A streptavidin-biotinylated HRP-based detection system was used to reveal specific binding. The sections were counterstained for 2 min at room temperature with hematoxylin for light microscopic review and evaluation. The expression was ranked on the sum of intensity and area from 0 to 7: 0-2, negative expression; 3-7, positive staining (of those, 3-4, weak positive expression; and 5-7, strong positive expression). Staining intensity was graded as follows: 0 for no staining; 1 for mild staining; 2 for moderate staining; and 3 for intense staining. The staining area was scored as follows: 0 for no staining; 1 for 1-25% area; 2 for 26-50% area; 3 for 51-75% area; and 4 for 76-100% area.

Statistical analysis. Statistical analysis was performed using SPSS 22.0 software (IBM Corp). The data were presented as the mean \pm standard deviation and measured using the Student's t-test (paired t-tests were used in Fig. 1C, and unpaired t-tests were used in Fig. 2C-F). ANOVA analysis followed by Dunnett's post hoc test was used for 2G-I. Chi-square test was used to analyze the association of circRNA expression with the hub protein expression (Fig. 7C). Kaplan-Meier analysis was used to evaluate the overall survival and log-rank test was performed to estimate the differences among the various groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Identification of differentially expressed circRNAs. The GSE101586 dataset from GEO was analyzed using the limma package in R ($P < 0.05$ and $|\log_2 FC| > 1$). In total, 47 upregulated and 21 downregulated circRNAs were identified in LAC tissues compared with adjacent non-cancerous tissues (Table I). After tightening the parameters to $P < 0.01$ and $|\log_2 FC| > 2$, four circRNAs, hsa_circ_0043278, hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666, were identified to be significantly upregulated in LAC tissues compared with adjacent non-cancerous tissues (Fig. 1A). The basic structural profiles of these four circRNAs are shown in Fig. 1B. All four of these circRNAs are derived from exons of their parent genes. Among them, three circRNAs (hsa_circ_0006220,

Table I. The differential expressed circRNAs between LAC tissues and the corresponding normal lung tissues.

CircRNA ID	P-value	Log ₂ (fold-change)
hsa_circ_0043278	0.002602	2.654576
hsa_circ_0006220	0.007278	2.567267
hsa_circ_0072088	0.001979	2.513804
hsa_circ_0000977	0.023568	2.450922
hsa_circ_0001666	0.008187	2.450695
hsa_circ_0022383	0.005301	1.898136
hsa_circ_0046263	0.00016	1.669339
hsa_circ_0000514	0.021638	1.553487
hsa_circ_0022392	0.016906	1.491607
hsa_circ_0005397	0.008214	1.480409
hsa_circ_0069086	0.006373	1.466104
hsa_circ_0036287	0.005599	1.460223
hsa_circ_0082564	0.0107	1.442822
hsa_circ_0027089	0.028481	1.394235
hsa_circ_0065214	0.005769	1.393836
hsa_circ_0018909	0.03837	1.389946
hsa_circ_0001998	0.041625	1.340807
hsa_circ_0004104	0.017903	1.324693
hsa_circ_0000519	0.02214	1.267825
hsa_circ_0003838	0.039573	1.267251
hsa_circ_0011385	0.01096	1.251054
hsa_circ_0002360	0.018803	1.249496
hsa_circ_0008583	0.035512	1.17612
hsa_circ_0055033	0.039073	1.169861
hsa_circ_0091710	0.022575	1.161549
hsa_circ_0067934	0.007101	1.143916
hsa_circ_0008539	0.047705	1.143884
hsa_circ_0084443	0.047543	1.136258
hsa_circ_0003028	0.03367	1.133201
hsa_circ_0017639	0.023814	1.123251
hsa_circ_0084429	0.021075	1.114951
hsa_circ_0072430	0.025704	1.103268
hsa_circ_0003958	0.024807	1.100155
hsa_circ_0001238	0.034705	1.099251
hsa_circ_0003528	0.034148	1.09902
hsa_circ_0040809	0.040398	1.077003
hsa_circ_0067971	0.03012	1.075765
hsa_circ_0007345	0.041766	1.075431
hsa_circ_0008274	0.038372	1.063426
hsa_circ_0025201	0.008555	1.062317
hsa_circ_0017109	0.011975	1.058244
hsa_circ_0069152	0.031401	1.051094
hsa_circ_0006948	0.028476	1.037828
hsa_circ_0000690	0.030795	1.033918
hsa_circ_0083054	0.041198	1.023474
hsa_circ_0005962	0.046675	1.002101
hsa_circ_0062389	0.04119	1.00164
hsa_circ_0005394	0.025027	-1.12294
hsa_circ_0005139	0.035675	-1.16601
hsa_circ_0092367	0.048694	-1.17796
hsa_circ_0076092	0.028292	-1.18494
hsa_circ_0000662	0.03011	-1.23507

Table I. Continued.

CircRNA ID	P-value	Log ₂ (fold-change)
hsa_circ_0061749	0.01061	-1.24893
hsa_circ_0001644	0.011159	-1.29063
hsa_circ_0002404	0.021288	-1.29969
hsa_circ_0001936	0.006073	-1.32711
hsa_circ_0000979	0.027511	-1.32785
hsa_circ_0029426	0.000788	-1.32894
hsa_circ_0000253	0.015088	-1.3675
hsa_circ_0030569	0.006596	-1.37248
hsa_circ_0003162	0.001318	-1.44889
hsa_circ_0031027	0.044118	-1.49911
hsa_circ_0043256	0.029359	-1.51818
hsa_circ_0019390	0.007575	-1.58187
hsa_circ_0007518	0.000301	-1.76043
hsa_circ_0049271	0.037986	-1.78724
hsa_circ_0076798	0.000399	-1.81001
hsa_circ_0015278	0.013596	-1.81839

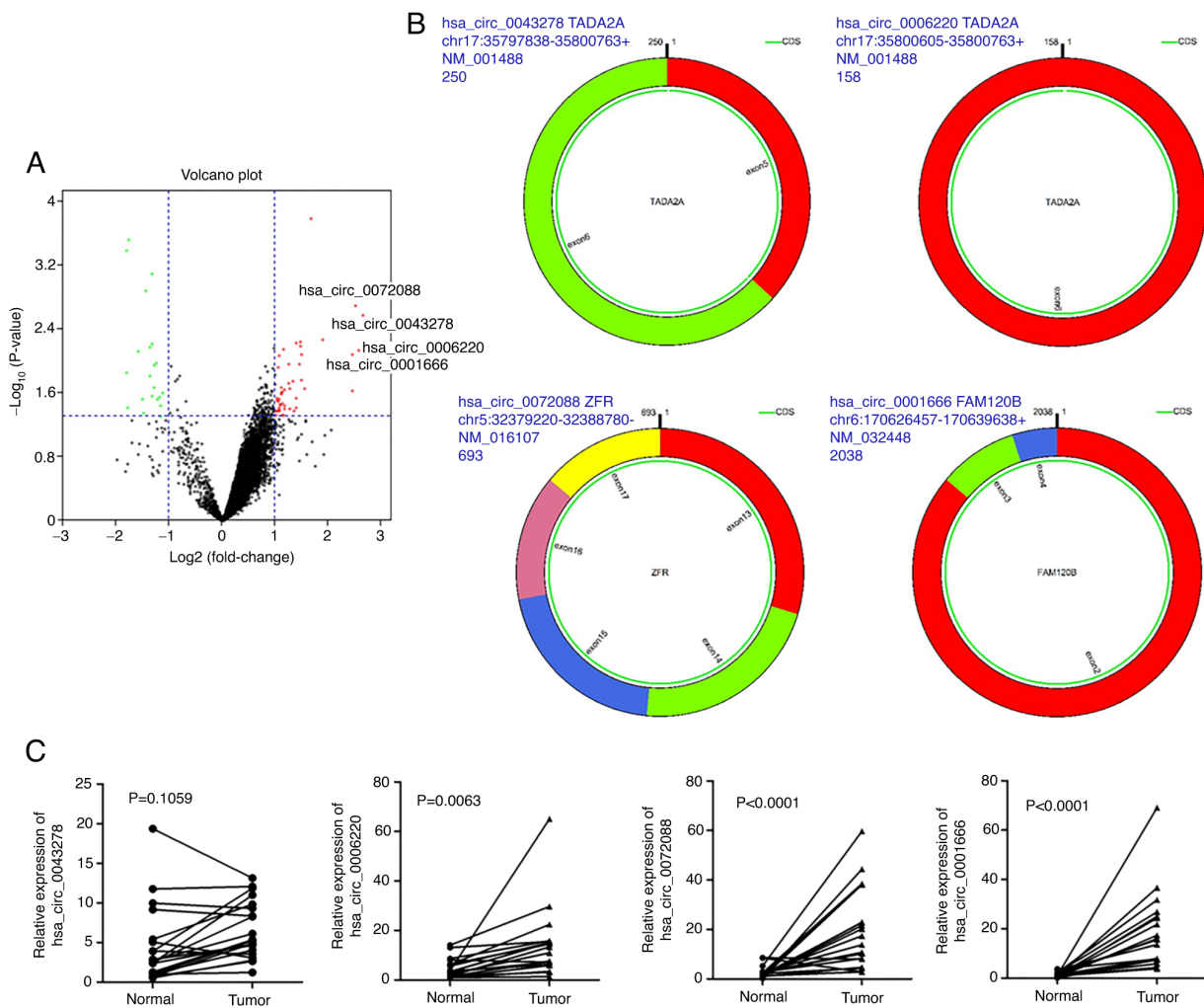


Figure 1. Differentially-expressed circRNAs between LAC tissues and the paired-matched adjacent normal lung tissues according to the GSE101586 dataset. (A) The differentially-expressed circRNAs are displayed in the volcano plot. (B) Schematic presentation of hsa_circ_0043278, hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666. (C) The expression of circRNAs in LAC tissues and the corresponding normal tissues detected by reverse transcription-quantitative PCR. circRNA, circular RNA; LAC, lung adenocarcinoma.

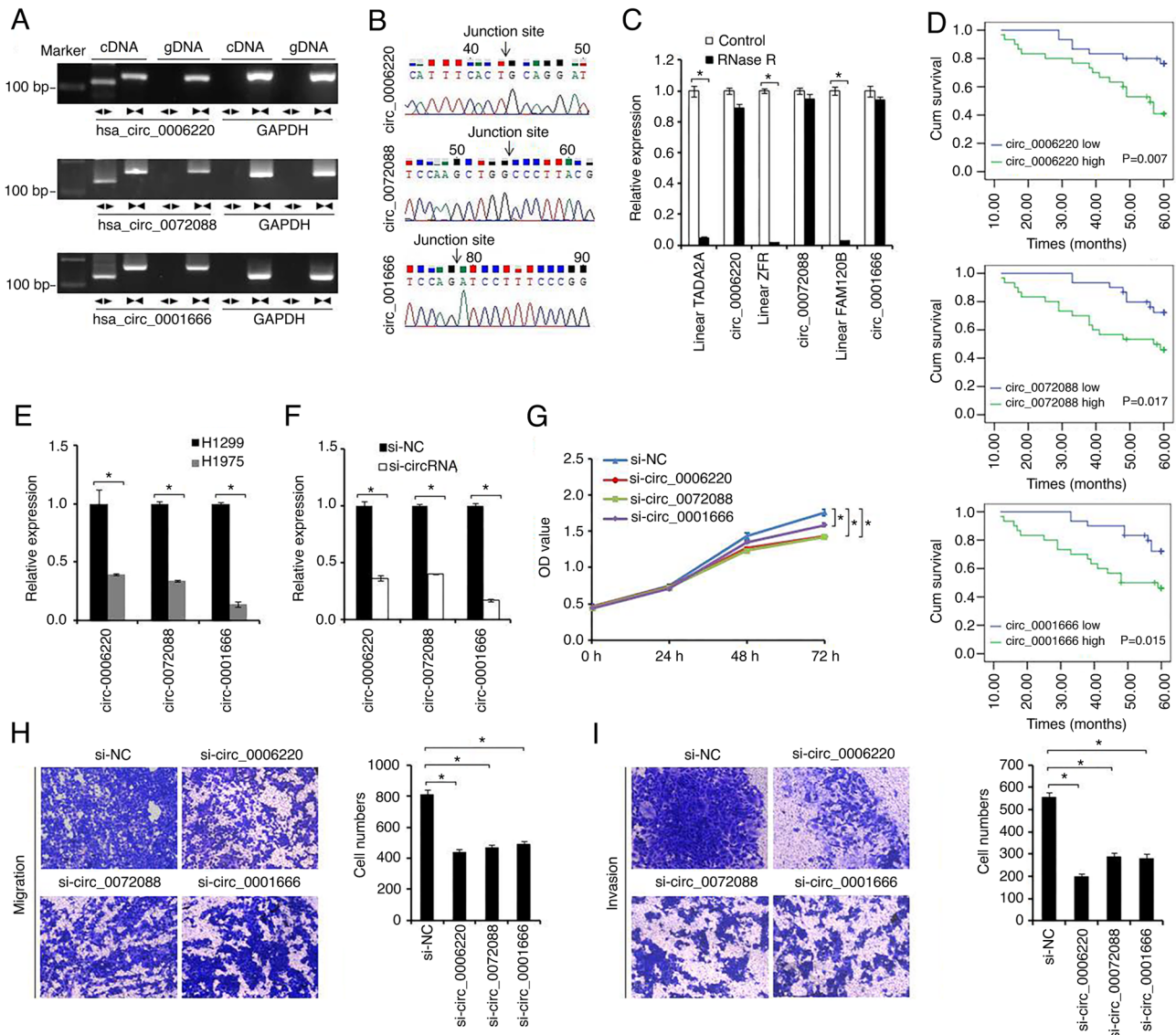


Figure 2. Hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 may serve oncogenic roles in LAC progression. (A) The expression of hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 was validated in LAC tissues by RT-qPCR. Divergent primers can amplify circRNAs in the cDNA but not gDNA. Convergent primers can amplify linear RNAs in both cDNA and gDNA samples. GAPDH was used as the negative control. (B) The sequence of the PCR product revealed the junction sites of the circRNAs. (C) All three of the circRNAs were resistant to RNase R treatment. (D) The association between the expression of these three circRNAs with the survival of 60 patients with LAC. (E) The expression of the three circRNAs in the LAC cell lines was measured using RT-qPCR. (F) The knockdown efficiency of the circRNAs siRNAs in H1299 cells. (G) Cell viability of H1299 cells was evaluated using MTT assay. * $P < 0.05$. H1299 cell (H) migration (magnification, $\times 100$) and (I) invasion (magnification, $\times 100$) was evaluated by Transwell migration and Matrigel invasion assays. * $P < 0.01$. circRNA, circular RNA; LAC, lung adenocarcinoma; RT-qPCR, reverse transcription-quantitative PCR; cDNA, complementary DNA; gDNA, genomic DNA; si, small-interfering.

hsa_circ_0072088, hsa_circ_0001666) were confirmed to be upregulated in LAC tissues compared with those in the corresponding normal tissues by RT-qPCR (Fig. 1C).

Survival analysis and biological functions of the four differentially-expressed circRNAs. To further explore the characteristics of three differentially-expressed circRNAs, two sets of primers for each circRNA were first designed. Divergent primers were expected to amplify the circular forms of the RNAs, whereas convergent primers were expected to amplify the linear forms of RNAs. Using cDNA and genomic DNA (gDNA) from LAC tissues as templates, all three of the differentially-expressed circRNAs were amplified by the divergent primers in cDNA, but no amplification products

were observed in the gDNA samples (Fig. 2A). All three linear forms of the circRNAs were amplified in both cDNA and gDNA samples. The sequences of the PCR products from all three circRNAs were verified by sequencing (Fig. 2B). By using RT-qPCR, it was verified further that all three of these circRNAs were resistant to RNase R, whilst the levels of their corresponding linear forms were significantly reduced after RNase R treatment (Fig. 2C).

Subsequently, the expression of these three circRNAs were observed in the 60 LAC tissue samples. In addition, higher expression levels of hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 indicated poor prognosis in patients with LAC (Fig. 2D). To further explore the biological functions of these differentially-expressed circRNAs, their expression was

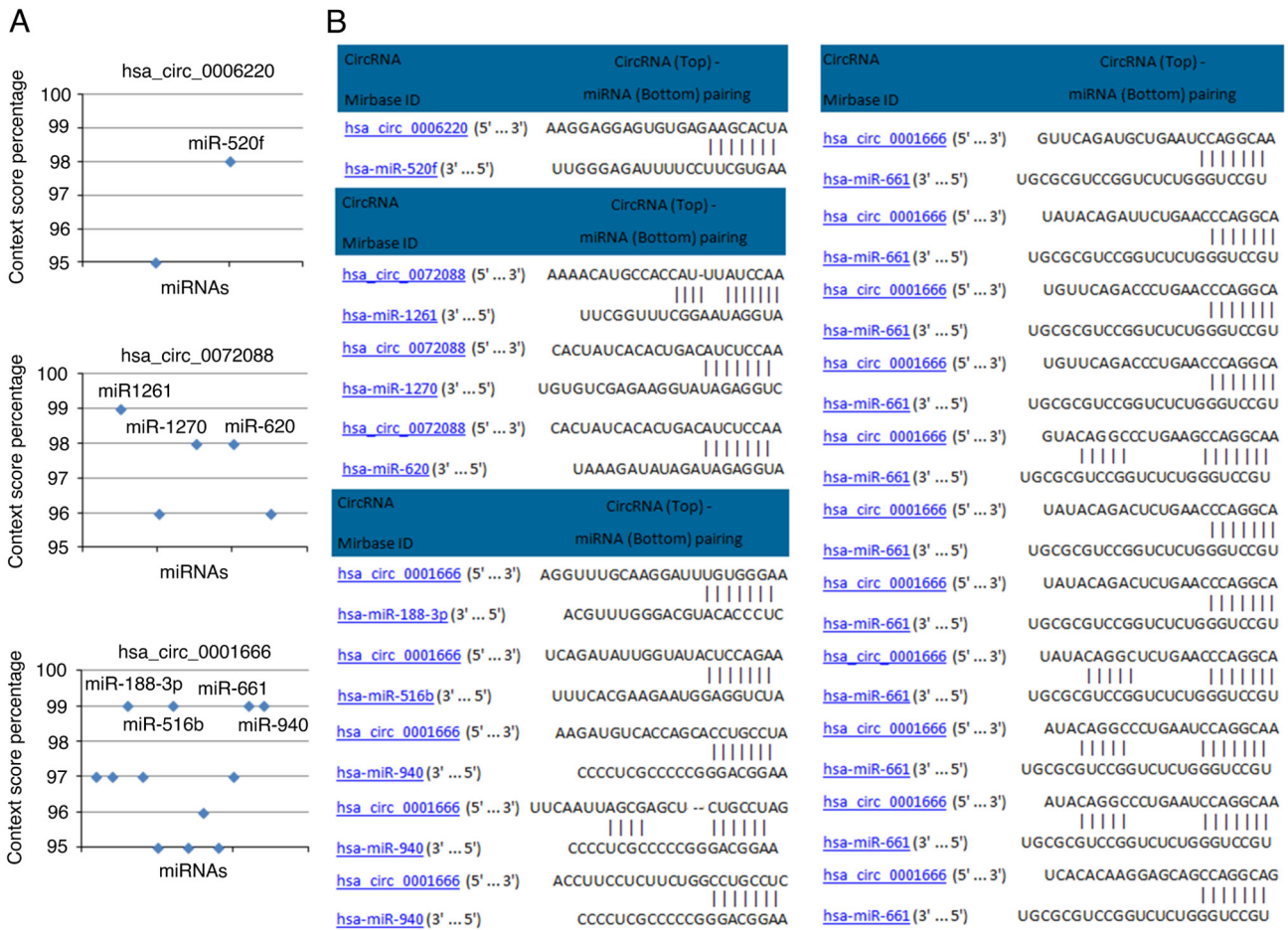


Figure 3. Identification of miRNAs that can potentially interact with the three circRNAs. (A) Screening of miRNAs that can interact with the three circRNAs using the CircInteractome database. (B) The potential binding sites between the circRNAs and miRNAs. miRNA, microRNA; circRNA, circular RNA.

then measured in the LAC cell lines (Fig. 2E). siRNA-mediated knockdown of these circRNAs was found to suppress proliferation, migration and invasion in LAC cells (Fig. 2F-I). Taken together, these results suggest that hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 can serve oncogenic roles in LAC progression and have potential as biomarkers and therapeutic targets for LAC.

Identification of miRNAs that can interact with the circRNAs. Accumulating evidence has demonstrated that several circRNAs derived from exons can exert biological functions by acting as ‘sponges’ or ‘decoys’ to sequester miRNAs. To assess if these three aforementioned circRNAs performed similar functions in LAC, miRNAs that can potentially interact with these three circRNAs were screened using the CircInteractome database. In total, eight miRNAs (miR-520f, miR-1261, miR-1270, miR-620, miR-188-3p, miR-516b, miR-940 and miR-661) with context score percentiles ≥ 98 were selected (Fig. 3A). The predicted binding sites between the circRNAs and miRNAs of interest are shown in Fig. 3B.

Construction of the circRNA-miRNA-mRNA regulatory network in LAC. A total of 7,829 target genes of the eight aforementioned miRNAs were obtained from the miRWalk software. Additionally, 795 upregulated genes in LAC were obtained from TCGA, according to the thresholds of false

discovery rate value <0.01 and $\text{Log}_2|\text{FC}|>3$ (Fig. 4A). By overlapping the predicted target genes and the upregulated genes in LAC, 232 target genes that may serve key roles in LAC were identified (Fig. 4B). From this, a circRNA-miRNA-mRNA network was constructed by integrating the circRNA-miRNA interactions and miRNA-mRNA interactions (Fig. 4C), which provided a preliminary insight into the links among the three differentially-expressed circRNAs (hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666), their eight suspected target miRNAs (miR-520f, miR-1261, miR-1270, miR-620, miR-188-3p, miR-516b, miR-940 and miR-661) and the 232 mRNAs.

PPI network construction and module selection. After removing the unconnected nodes, a PPI network consisting of 158 nodes and 287 edges was constructed to view the interaction among the 232 target genes (Fig. 5A). Subsequently, a pivotal module of six hub genes, kinesin family member (KIF) 2C, KIF18B, maternal embryonic leucine zipper kinase (MELK), baculoviral IAP repeat-containing 5 (BIRC5), polo-like kinase 1 (PLK1) and cytoskeleton-associated protein 2-like (CKAP2L), were identified from the PPI network using the CytoHubba plugin in Cytoscape (Fig. 5B).

Survival analysis of the six hub genes. The six hub genes in the PPI network were next evaluated for their expression

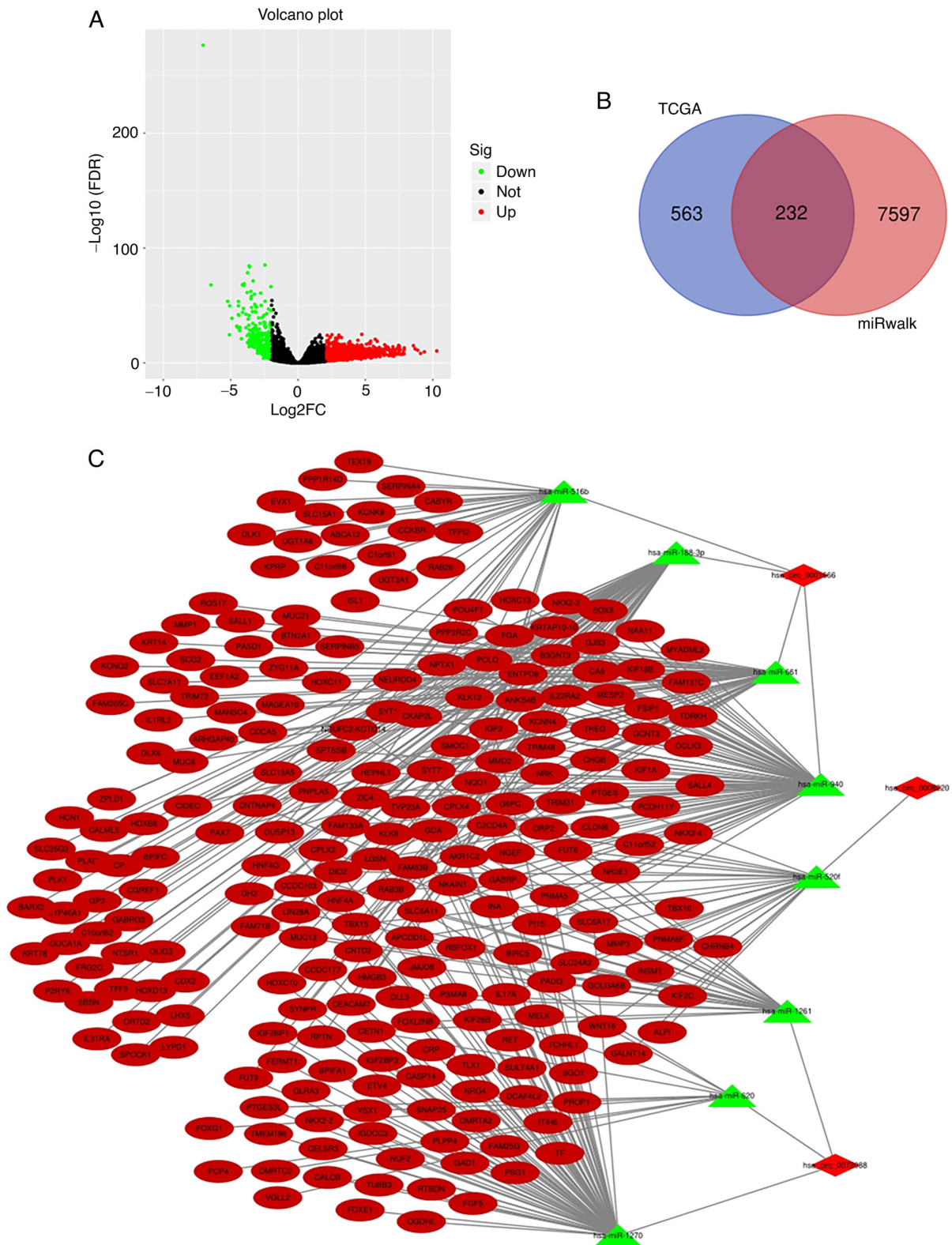


Figure 4. Construction of the circRNA-miRNA-mRNA regulatory network for LAC. (A) Volcano plot showing differentially-expressed genes between LAC and normal lung tissues in TCGA. (B) Venn diagram showing 232 overlapping differentially-expressed genes identified from TCGA and targeted mRNAs from miRWalk. (C) The ceRNA regulatory network of circRNAs-miRNAs-mRNAs in LAC. Diamonds represent circRNAs, triangles represent miRNAs and ovals represent mRNAs. circRNA, circular RNA; miRNA, microRNA; LAC, lung adenocarcinoma; TCGA, The Cancer Genome Atlas database; ceRNA, competing endogenous RNAs.

levels and prognostic value using the GEPIA database. As shown in Fig. 6A-F, the expression of these six hub genes in LAC tissues was significantly higher compared with that in

the normal tissues. All of these six hub genes exhibited their potential in the prediction of overall survival based on their expression. High expression levels of each hub gene were

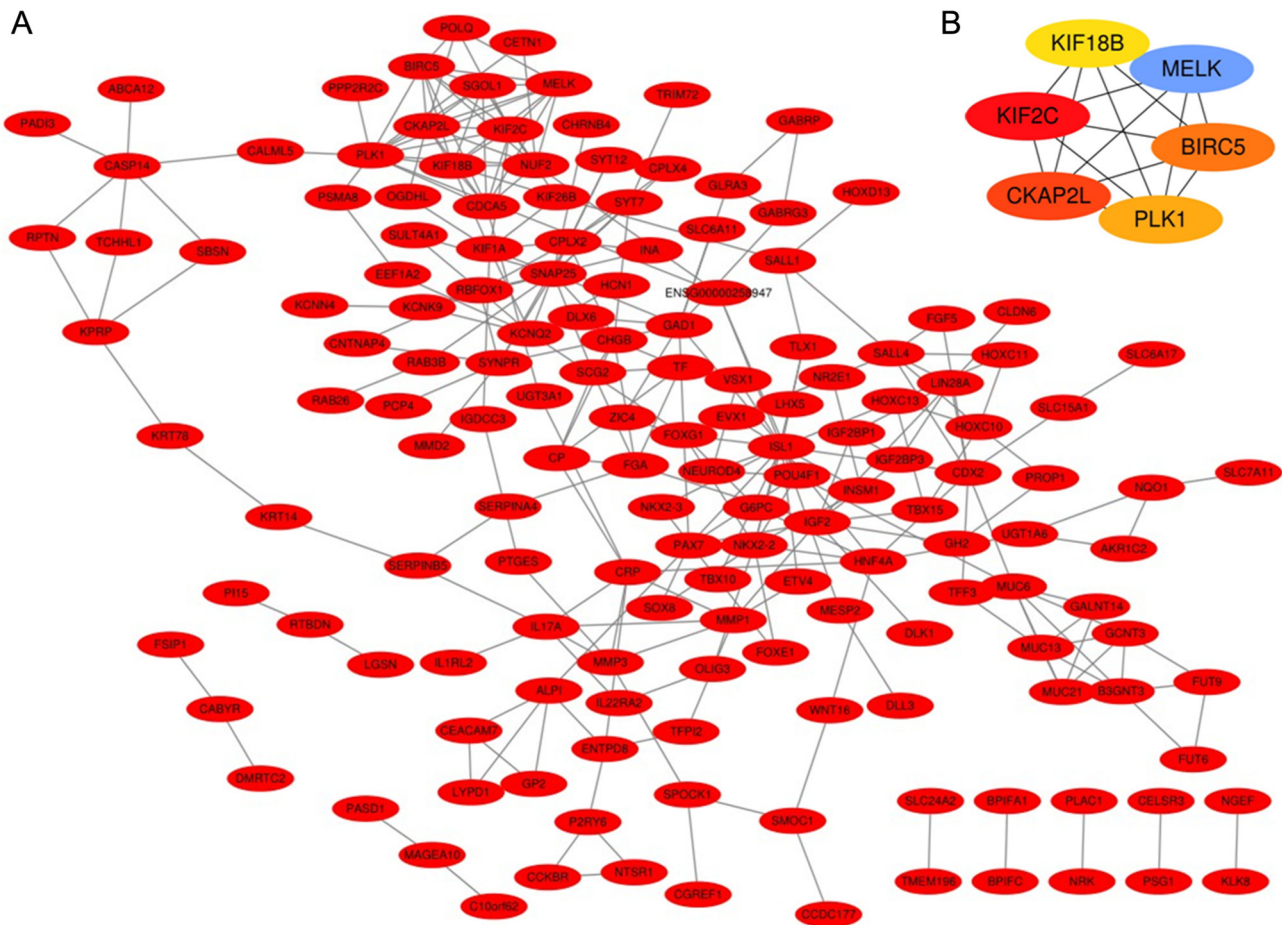


Figure 5. PPI network construction and screening for hub genes. (A) A PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins database and visualized using the Cytoscape software. (B) The top six hub genes were selected from the PPI network using the CytoHubba plugin in Cytoscape. PPI, protein-protein interaction.

associated with poorer overall survival in patients with LAC (Fig. 6A-F).

A circRNA-miRNA-hub gene sub-network was then built to delineate the links among the differentially-expressed circRNAs, target miRNAs and hub genes (Fig. 6G). Of note, the hsa_circ_0006220/hsa-miR-520f/KIF2C, hsa_circ_0001666/hsa-miR-940/KIF18B, hsa_circ_0001666/hsamiR-940/CKAP2L, hsa_circ_0001666/hsa-miR-661/CKAP2L, hsa_circ_0001666/hsa-miR-188-3p/PLK1, hsa_circ_0072088/hsamiR-1270/MELK and hsa_circ_0072088-hsa-miR-1261-BIRC5 regulatory axes were found from this network. Taken together, these results suggest that hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 may serve oncogenic roles by regulating the expression of these six hub genes.

Validation in clinical samples. To further validate the findings, immunohistochemistry was performed in the 60 LAC samples. The statistical results showed that high expression levels of KIF2C, KIF18B, MELK, BIRC5, PLK1 and CKAP2L were associated with the poorer survival in patients with LAC (Fig. 7A and B), which was consistent with the results of GEPIA database analysis. In addition, the expression of hsa_circ_0006220 was positively correlated with the expression of KIF2C, whereas the expression of hsa_circ_0001666 was

positively correlated with the expression of KIF18B, MELK and BIRC5. The expression of hsa_circ_0072088 was positively correlated with the expression of PLK1 and CKAP2L (Fig. 7C).

Discussion

Accumulating evidence indicates that circRNAs are stable, abundant and highly conserved in eukaryotic cells, with high disease specificity (18-20). In lung cancer, an increasing number of circRNAs, including circ-calcium/calmodulin dependent protein kinase II α (21), circ-solute carrier family 25 member 16 (22), circ-SATB homeobox 2 (23), circ-ATP-binding cassette subfamily B member 10 (24), circHMCU (25) and circ-phosphatidylinositol-4-phosphate 5-kinase type 1 α (26), have been reported to serve regulatory roles in disease progression. These previous findings suggest that circRNAs are suitable markers for guiding clinical diagnosis and therapy. However, a large number of potentially beneficial circRNAs remain undiscovered.

In the present study, a GEO dataset was analyzed to investigate the differentially-expressed circRNAs in LAC, which found four circRNAs (hsa_circ_0043278, hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666) to be highly expressed in LAC tissues. These candidate circRNAs may be involved in LAC pathogenesis and can serve as the

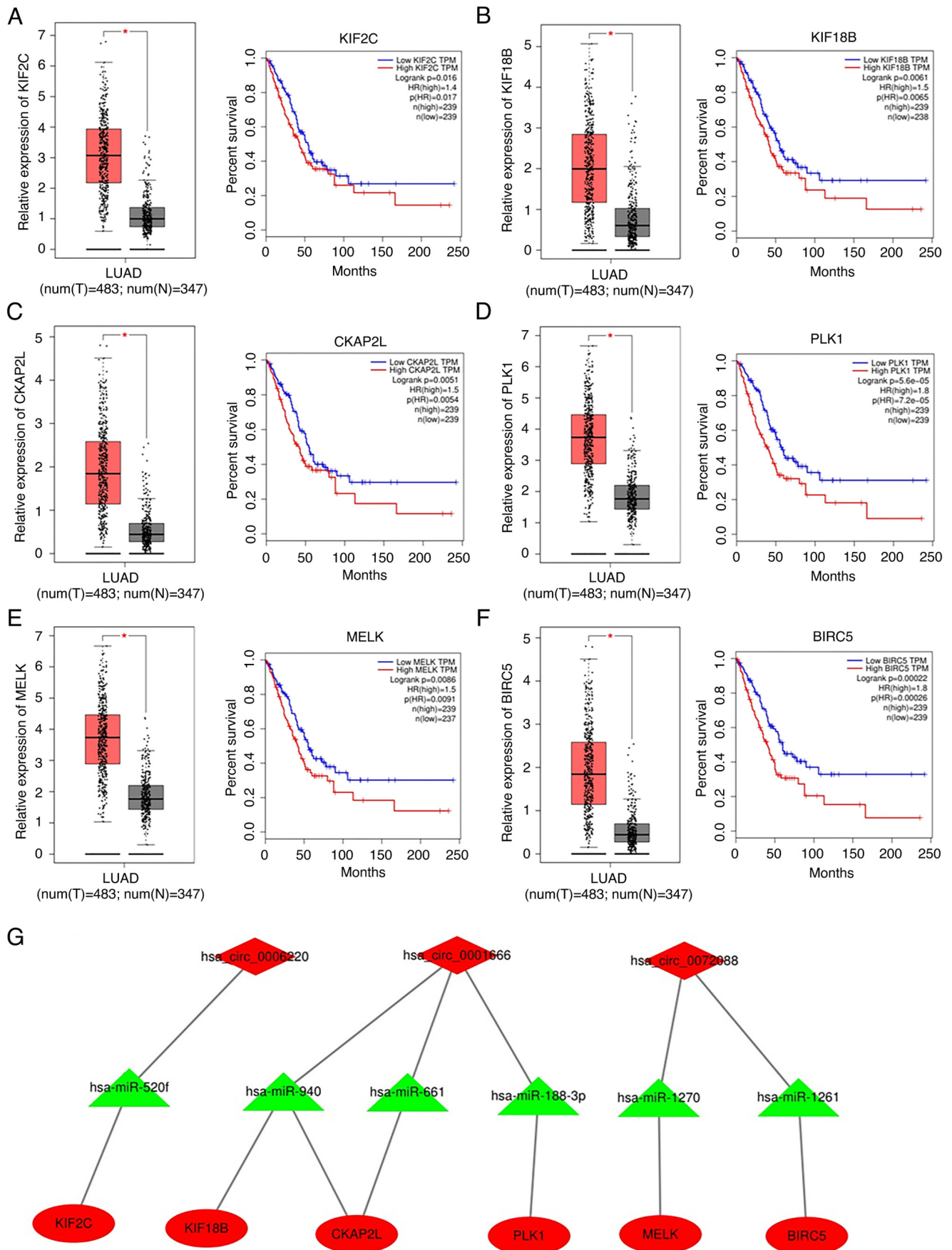


Figure 6. Relative expression and survival analysis of the six hub genes in LAC using the GEPIA database. Expression of (A) KIF2C, (B) KIF18B, (C) cytoskeleton-associated protein 2-like, (D) polo-like kinase 1, (E) maternal embryonic leucine zipper kinase and (F) and baculoviral IAP repeat-containing 5 was measured. Red color represents tumor tissues, and gray color represents normal tissues. (G) The circRNA-miRNA-hubgene sub-network was constructed. Diamonds represent circRNAs, triangles represent miRNAs and ovals represent mRNAs. * $P < 0.05$. LAC, lung adenocarcinoma; GEPIA, Gene Expression Profiling Interactive Analysis; KIF, kinesin family member; circRNA, circular RNA; miRNA, microRNA.

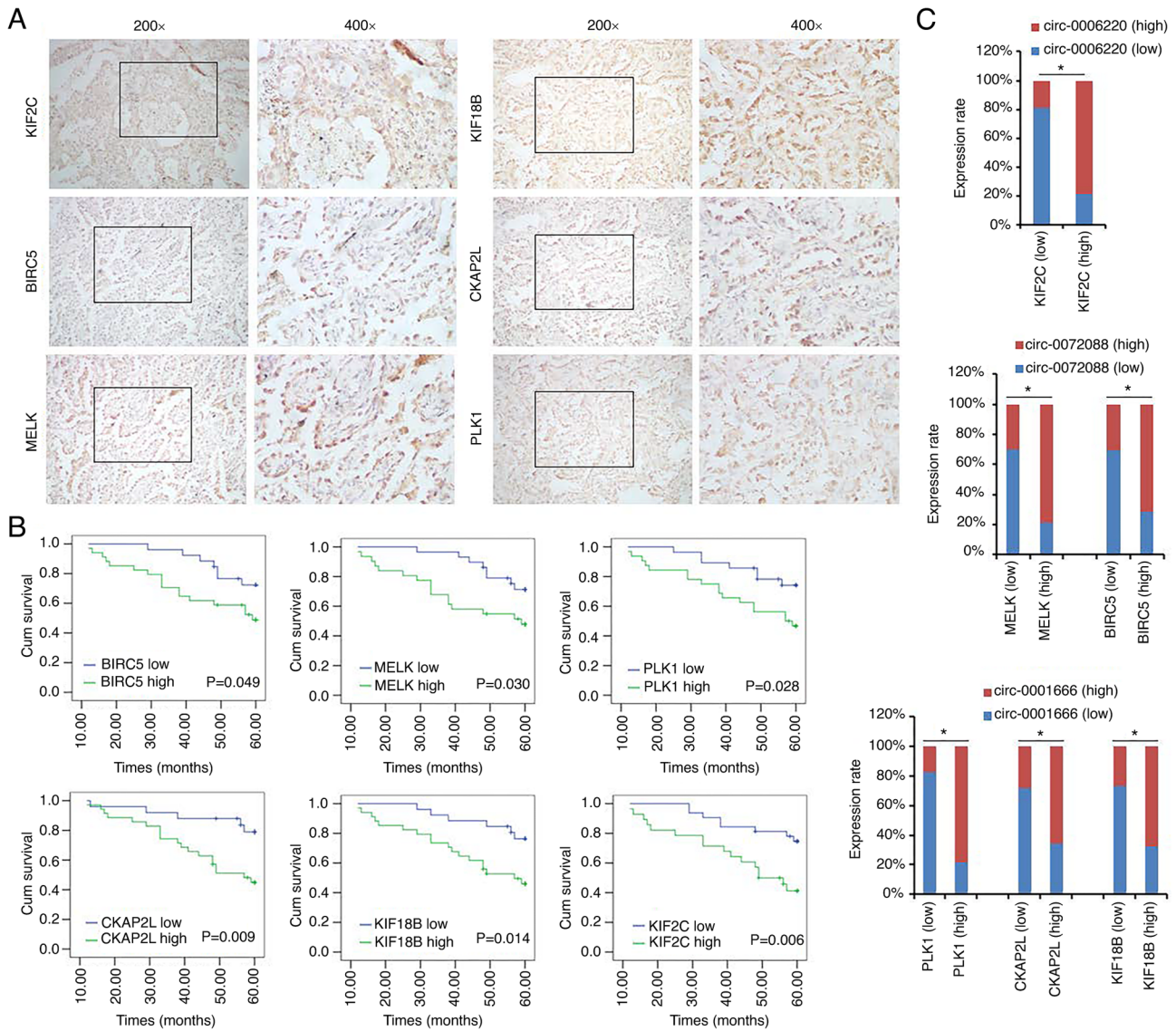


Figure 7. Validation of hub genes in clinical samples. (A) Expression and (B) survival analysis of the six hub genes in patient samples of lung adenocarcinoma. Left magnification, x200; Right magnification, x400. (C) Association between the expression of the circular RNAs and hub genes. *P<0.05. KIF, kinesin family member.

biomarkers for targeted therapy. By analyzing their expression in 20 paired LAC tissues and adjacent normal lung tissues, it was found that the expression levels of hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 were markedly higher in LAC tissues, where high expression levels of these three circRNAs indicated poorer prognosis in patients with LAC. In addition, siRNA-mediated knockdown of these three circRNAs suppressed the proliferation, migration, and invasion of LAC cell lines, suggesting their potential oncogenic functions in LAC. These results suggest that hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 can serve oncogenic roles in LAC cells.

As conserved endogenous RNAs, circRNAs can sponge miRNAs and serve key roles in regulating the expression of the target genes of miRNAs. To explore the sponged miRNAs of these three aforementioned circRNAs, the CircInteractome online database (27,28) was used to predict the miRNAs that have the ability to bind these circRNAs. In total, eight miRNAs (miR-520f, miR-1261, miR-1270, miR-620, miR-188-3p, miR-516b, miR-940 and miR-661) were determined to have

high potential to bind these circRNAs. Subsequently, potential mRNAs targeted by these miRNAs were predicted using miRWalk before a ceRNA network was constructed.

To verify the action of this ceRNA network, a PPI network was next constructed, which yielded six hub genes, namely KIF2C, KIF18B, MELK, BIRC5, PLK1 and CKAP2L. The oncogenic roles of these six genes in cancer have also been demonstrated by previous studies (29-39). In the present study, the prognostic value of these hub genes was also analyzed in LAC based on the GEPIA database. High expression levels of each hub gene were associated with a poorer overall survival in patients with LAC. The *in vivo* experiments showed that high expression levels of KIF2C, KIF18B, MELK, BIRC5, PLK1 and CKAP2L were associated with poorer survival in patients with LAC, which was in accordance with the results from the GEPIA database. In addition, the expression of hsa_circ_0006220 was positively correlated with the expression of KIF2C, whereas the expression of hsa_circ_0001666 was positively correlated with the expression of KIF18B, MELK and BIRC5. The expression of hsa_circ_0072088 was positively

correlated with the expression of PLK1 and CKAP2L. These results suggested that hsa_circ_0006220, hsa_circ_0072088 and hsa_circ_0001666 may serve oncogenic roles in LAC by regulating the expression of these hub genes. However, further studies are required to confirm that the circRNAs affect the expression of the proteins encoded via the above six miRNAs.

As there is no public circRNA data in TCGA database, the GEO database was chosen to screen for the target circRNAs in LAC, which provided guidance for the follow-up validation and experiments. The retrospective clinical study and biological experiments confirmed the oncogenic function of these selected circRNAs. The ceRNA and PPI networks were then constructed using bioinformatic analysis, using which the hub genes were revealed and partially explained the molecular mechanism underlying the aforementioned circRNAs. However, further experiments are required to validate these findings. Conclusively, through the bioinformatic analysis and retrospective clinical study, results from the present study provided novel circRNAs biomarkers and therapeutic targets for LAC. Further experiments in animals would be useful to validate these findings.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BS and MS contributed to the conception and design of the work. YZ, FC, SL, LM, LG performed the experiments. FL and HZ analyzed the data and performed the bioinformatical analysis. YZ and BS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethical approval and consent to participate

The human tissues were obtained with informed consent and the present study was approved by The Clinical Research Ethics Committee of The Fourth hospital of Hebei Medical University (approval no. 2021KY157).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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